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Expression of the *gltP* gene of *Escherichia coli* in a glutamate transport-deficient mutant of *Rhodobacter sphaeroides* restores chemotaxis to glutamate

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Summary

***Rhodobacter sphaeroides* is chemotactic to glutamate and most other amino acids. In *Escherichia coli*, chemotaxis involves a membrane-bound sensor that either binds the amino acid directly or interacts with the binding protein loaded with the amino acid. In *R. sphaeroides*, chemotaxis is thought to require both the uptake and the metabolism of the amino acid. Glutamate is accumulated by the cells via a binding protein-dependent system. To determine the role of the binding protein and transport in glutamate taxis, mutants were created by Tn*5* insertion mutagenesis and selected for growth in the presence of the toxic glutamine analogue γ-glutamyl-hydrazide. One of the mutants, *R. sphaeroides* MJ7, was defective in glutamate uptake but showed wild-type levels of binding protein. The mutant showed no chemotactic response to glutamate. Both glutamate uptake and chemotaxis were recovered when the *gltP* gene, coding for the H^+-linked glutamate carrier of *E. coli*, was expressed in *R. sphaeroides* MJ7. It is concluded that the chemotactic response to glutamate strictly requires uptake of glutamate, supporting the view that intracellular metabolism is needed for chemotaxis in *R. sphaeroides*.**

Introduction

Methyl-accepting chemotaxis proteins (MCPs) are found in many bacteria. MCPs are located in the cytoplasmic membrane and enable the bacterium to monitor the composition of its chemical surrounding. Attractants and some repellents bind to the periplasmic domain of the transducer. Ligand binding may induce a conformational change in the periplasmic domain of the transducer that propagates through the membrane to the cytoplasmic domain. Cells respond to attractants and repellents through phosphorylation and dephosphorylation and adapt through methylation and demethylation of specific glutamate residues in the cytoplasmic domain of the transducer (Stock *et al.*, 1989). This system is not dependent on transport of the attractant but only requires binding of the attractant to MCPs. Chemotactic sensing in the purple phototrophic bacterium *Rhodobacter sphaeroides* is very different from that in enteric bacteria. *R. sphaeroides* lacks MCPs as well as the phosphotransferase-system-dependent chemotaxis systems found in enteric bacteria (Armitage, 1992; Sockett *et al.*, 1987; Stock *et al.*, 1985). *R. sphaeroides* responds to a wide variety of stimuli, such as weak organic acids, weak bases, ions, oxygen and light (Armitage *et al.*, 1985; Armitage, 1992; Ingham and Armitage, 1987; Poole and Armitage, 1989). All identified chemoattractants are also metabolites (Armitage, 1992; Poole and Armitage, 1989; Poole *et al.*, 1993). Since both chemotaxis and active transport contribute to nutrient acquisition, it is of interest to determine whether taxis and uptake activities are co-ordinated in some manner. Indications of co-ordinated chemotaxis and uptake have been reported previously. Ingham and Armitage (1987) suggested that transport and metabolism of attractants was required for chemotactic sensing in *R. sphaeroides.* Competitive inhibitors of propionate uptake inhibit the chemotactic response to propionate (Ingham and Armitage, 1987), while cells show no chemotactic response to the non-metabolizing alanine analogue α-aminoisobutyrate (Poole *et al.*, 1993). The latter is transported via the alanine-binding protein-dependent system but is not metabolized. Metabolism of ammonia appears to be required for chemotaxis, as methionine sulphoximine, an inhibitor of incorporation of ammonia via the glutamine synthetase-dependent pathway, blocks chemotaxis (Poole and Armitage, 1989). These data, albeit indirectly, suggest a requirement for transport and metabolism for chemotaxis.

Many, if not all, amino acids to which *R. sphaeroides* shows a chemotactic response (Packer and Armitage, 1994) are transported by binding protein-dependent systems (Abee, 1989; Jacobs *et al.*, 1995). We have recently characterized a binding protein-dependent system for glutamate and glutamine (Jacobs *et al.*, 1995). To obtain more

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direct data supporting the view that transport is needed for chemotaxis, we have isolated by transposon mutagenesis a mutant defective in glutamate transport. This mutant is also defective in chemotaxis to glutamate. Both the chemotactic response and the uptake of glutamate are recovered when the H^+-linked glutamate transport system of *Escherichia coli* is expressed in an *R. sphaeroides* mutant. It is concluded that, for chemotaxis to glutamate, uptake of this amino acid is an obligatory requirement.

Results

Isolation of a glutamate transport-deficient mutant of R. sphaeroides

Glutamate transport-deficient mutants were selected by plating transposon mutants on Sistrom medium containing the toxic glutamine analogue γ-glutamyl-hydrazide. Colonies growing on these plates were further screened for growth on 20 mM glutamate as sole carbon and nitrogen source. Colonies unable to grow on this medium were tested for the uptake activity of glutamate and proline. One of the mutants, *R. sphaeroides* MJ7, showed a very low glutamate uptake activity compared with the wild type at a glutamate concentration of 0.94 μM (Fig. 1A) or a higher concentration of 10 mM (Fig. 1B). The uptake of proline was not affected in this mutant (data not shown). Immunoblot analysis indicated that mutant cells contained nearly the amount of glutamate-binding protein as the wild type, while the shock fluid derived from the mutant cells was still able to bind [^{3}H-] glutamate (data not shown). These data indicate that *R. sphaeroides* MJ7 has a deficiency in glutamate transport.

Expression of the H^+-linked glutamate carrier of E. coli *in* R. sphaeroides

To demonstrate that the inability of *R. sphaeroides* MJ7 to grow on glutamate results from a defective transport of glutamate, the *gltP* gene coding for the H^+-linked glutamate-transport protein of *E. coli* was introduced into *R. sphaeroides* MJ7. Plasmid pGBT521 (Tolner *et al.*, 1992; 1995) was digested with *Eco*RI and *Pst*I to yield an *Eco*RI–*Pst*I fragment containing the *gltP* gene with its own promoter. This fragment was ligated into the *Eco*RI and *Pst*I site of the expression vector pCHB500 (Benning and Somerville, 1992) to yield plasmid pMJ100 with *gltP* under control of the putative *R. capsulatus* promoter of cytochrome c_2 (Benning and Somerville, 1992). This plasmid was transformed to *E. coli* S17-1 and subsequently conjugated to the mutant *R. sphaeroides* MJ7. In this strain, termed MJ7GltP, *gltP* was functionally expressed, as demonstrated by the significant restoration of the uptake of glutamate (Fig. 1) and the restoration of growth on glutamate (data not shown). In contrast to the wild type (Fig. 2A), glutamate uptake activity of EDTA-treated cells of strain MJ7GltP was completely inhibited by simultaneous addition of the ionophores valinomycin and nigericin at pH 8.0 (Fig. 2B). These data are consistent with the conclusion that uptake of glutamate via GltP is proton motive-force driven (Tolner *et al.*, 1995), while glutamate uptake via the *R. sphaeroides* 4P1 L-glutamate transport system is ATP driven (Jacobs *et al.*, 1995). Strain MJ7GltP accumulated glutamate with an apparent affinity for transport of 11 μM. This value is close to that reported for GltP-dependent glutamate transport in *E. coli* (Deguchi *et al.*, 1989; Tolner *et al.*, 1995). These data demonstrate that *gltP* can be functionally expressed in *R. sphaeroides* and that the inability of strain MJ7 to grow on glutamate as

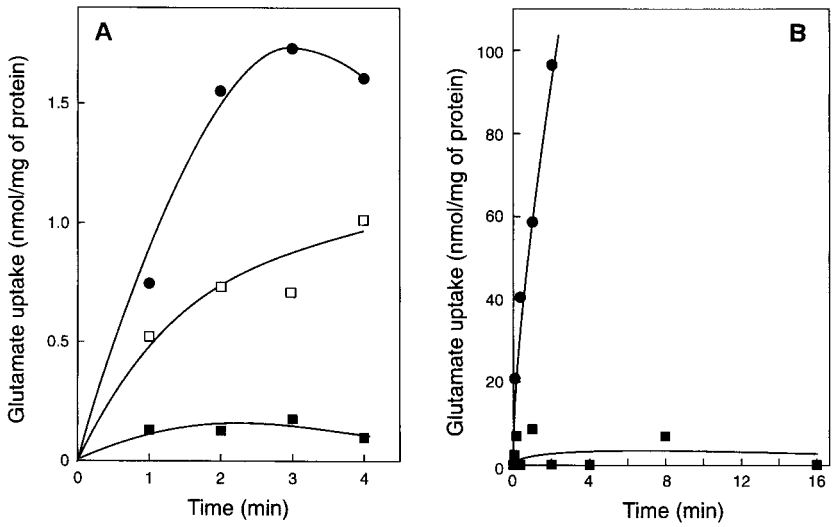

Fig. 1. L-Glutamate transport in *R. sphaeroides* 4P1 (●), MJ7 (■) and MJ7GltP (□). The glutamate concentration was 0.94 μM (A) or 10 mM (B).

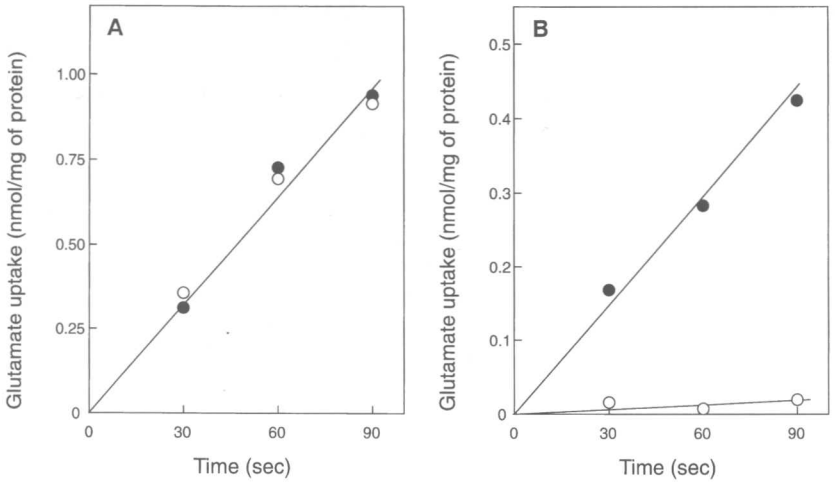

Fig. 2. Effect of the dissipation of the proton-motive force on L-glutamate uptake by *R. sphaeroides* 4P1 (A) and *R. sphaeroides* MJ7GltP (B). Uptake of L-glutamate by EDTA-treated cells of *R. sphaeroides* 4P1 or MJ7GltP was assayed in the absence (●) and presence (○) of nigericin plus valinomycin (4 μM each) at pH 8.0.

sole nitrogen and carbon source is the result of a deficiency of transport.

Transport is essential for taxis towards glutamate

Glutamate is a good chemoattractant for *R. sphaeroides* 4P1 (Fig. 3A). To investigate whether glutamate transport is important for chemotaxis, *R. sphaeroides* MJ7 cells were tested for the chemotactic response to glutamate. Although these cells still contain the glutamate-binding protein they are deficient in glutamate uptake (Fig. 1). Figure 3B shows that *R. sphaeroides* MJ7 is also deficient in glutamate taxis. In contrast, *R. sphaeroides* MJ7GltP expressing the H^+-linked glutamate transport protein of *E. coli* showed a normal chemotactic response to glutamate (Fig. 3C). The lack of taxis towards glutamate by *R. sphaeroides* MJ7 is not simply the result of energy deprivation as taxis to glutamate was also not observed when the energy source fructose (Lolkema *et al.*, 1986) was added to the agar (data not shown). In wild-type cells, fructose did not interfere with taxis towards glutamate (see Table 1). Both wild-type and mutant cells showed comparable taxis towards succinate (data not shown). These data demonstrate that transport of glutamate is essential for chemotaxis to glutamate but the mechanism by which transport occurs is not important. It furthermore indicates that the binding protein for glutamate plays only an indirect role in chemotaxis in as far it is needed for transport. These data exclude the possibility that the binding protein acts in the periplasm via a chemotaxis receptor.

Intermediates in glutamate metabolism inhibit chemotaxis

Under aerobic conditions, glutamate enters the tricarboxylic acid cycle as pyruvate. In addition, glutamate can serve as amino donor in a number of biosynthetic pathways. The chemotactic response may be mediated by one of the intermediates of the metabolic pathway. To analyse this, a series of possible intermediates and unrelated metabolites were mixed with the cells in the soft agar, and their effects on chemotaxis towards glutamate were determined

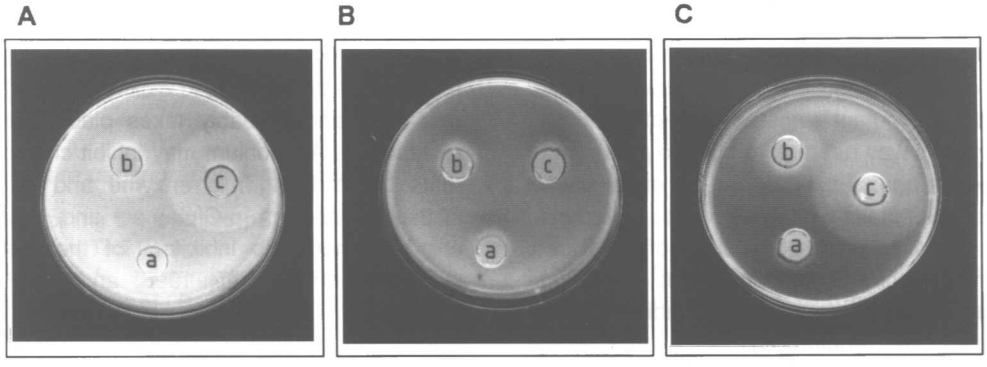

Fig. 3. Chemotactic response in plug plates of *R. sphaeroides* strains 4P1 (A), MJ7 (B) and MJ7GltP (C) to L-glutamate. Cells were included in the soft agar that contained 50 μg ml^{-1} chloramphenicol to inhibit growth. Hard plugs contained L-glutamate in concentrations of (a) 0, (b) 10 and (c) 50 mM.

Table 1. Effect of a range of compounds on the chemotactic response to L-glutamate of *R. sphaeroides* 4P1.

Competitor	Concentration (mM)	Inhibitory effect[a]
L-Alanine	20	+
L-Serine	20	+/−
L-Aspartate	20	+/−
D-Glutamate	20	+
α-Methylglutamate	20	+
γ-Aminobutyrate	20	−
Fructose	50	−
Acetate	50	−
Pyruvate	50	+
Succinate	50	+
Fumarate	50	+
Ammonium chloride	50	+

a. The chemotactic response to L-glutamate in the presence of a compound was compared with the response when these compounds were lacking. The symbols reflect the extent of inhibition: weak (−), intermediate (+/−) and strong (+).

(Table 1). Little effect of alternative carbon sources, such as fructose and acetate, on the chemotaxis towards glutamate was observed. However, pyruvate, succinate and fumarate, each intermediates of the tricarboxylic acid cycle, were strong inhibitors. Strong inhibition was also observed with alternative nitrogen sources, such as ammonium chloride and various amino acids. Although these experiments do not allow an identification of the metabolite that is sensed, the results do suggest that the chemotactic response of *R. sphaeroides* to glutamate is transduced through one of the intermediates in its metabolism.

Discussion

In this study, direct evidence is provided that the chemotactic response of *R. sphaeroides* towards glutamate involves transport of glutamate into the cell. The chemotactic response of the glutamate transport-deficient mutant *R. sphaeroides* MJ7 could be restored by the introduction of the *gltP* gene, coding for the H^+-linked glutamate transporter of *E. coli*. The introduction of this gene into *R. sphaeroides* MJ7 also restored the uptake of glutamate. The absence of a chemotactic response towards glutamate observed in the mutant is not the result of insufficient energization as taxis was not restored by the presence of fructose in the soft agar. Fructose had no effect on the chemotaxic response of the wild type towards glutamate.

Although chemotaxis requires transport of glutamate, the mechanism of transport seems irrelevant. In wild-type cells, glutamate is accumulated via a binding protein-dependent system that requires ATP as energy source (Jacobs *et al.*, 1995). In the mutant strain expressing *gltP*, glutamate uptake is driven by the proton motive force. At the concentration of glutamate tested, the chemotaxis sensitivity appeared to be the same for both transport systems. However, in the *in situ* situation, the high affinity of the binding protein-dependent transport system for glutamate, i.e. 1.2 μM compared with 11 μM for GltP, may be advantageous in allowing cells to sense low environmental concentrations of glutamate (Tempest *et al.*, 1970).

The need for transport and metabolism for chemotaxis in *R. sphaeroides* has been suggested before (Ingham and Armitage, 1987; Poole *et al.*, 1993) but the evidence has been only indirect. Analogues which inhibit transport are also known to inhibit chemotaxis. So far, only binding protein-dependent transport systems have been found in *R. sphaeroides*. Since both chemotaxis and transport involve a binding protein in the wild type, an alternative explanation for the inhibition of analogues could be that the binding protein–analogue complex is unable to interact with a sensor exposed to the periplasmic face of the cytoplasmic membrane. Only binding of the specific ligand to the binding protein induces a conformational change which allows the ligand–binding protein complex to interact with the appropriate sensor/transducer (Abouhamad *et al.*, 1991). Since the glutamate transport-deficient mutant strain still expressed appreciable amounts of active glutamate-binding protein but has lost the tactic behaviour toward glutamate, it is evident that the presence of binding protein is not sufficient for chemotaxis.

Chemotaxis in *R. sphaeroides* appears to involve the metabolism of the chemoattractant. For instance, cells show no chemotactic response to the non-hydrolysable alanine analogue α-aminoisobutyrate although this compound is rapidly accumulated by the cells (Poole *et al.*, 1993). To obtain some indications on the nature of the metabolite that transduces the chemotactic response of the organism to glutamate, the impact of several intermediates of glutamate metabolism and some alternative nitrogen sources on glutamate chemotaxis was studied. Ammonium is a strong inhibitor of the chemotactic response to L-glutamate but not to L-alanine and L-serine under conditions where cells are grown with excess nitrogen (Ingham and Armitage, 1987). Under the latter conditions, glutamate dehydrogenase is responsible for ammonium assimilation (Brown and Gibson, 1977). Glutamate can be deaminated to 2-oxoglutarate. This reaction is reversible and probably takes place during glutamate metabolism. Ammonium may inhibit chemotaxis to glutamate by inhibiting this enzyme and thereby blocking further metabolism. D-Glutamate and α-methylglutamate are both non-toxic inhibitors of the glutamate-uptake system (Jacobs *et al.*, 1995) and may, therefore, be transported.

Inhibition of chemotaxis can occur at two levels: by competition with glutamate for uptake or by inhibiting enzymes involved in glutamate metabolism. D-Glutamate may also be converted by a racemase into L-glutamate. An example

of a metabolite involved in chemotaxis is fumarate. This compound plays a role in chemotaxis in *Halobacterium halobium* and *E. coli* (Barak and Eisenbach, 1992; Marwan *et al.*, 1990). Fumarate also inhibits glutamate taxis in *R. sphaeroides* but is not likely to be the central metabolite in chemotaxis because it caused no exceptional responses in studies reported by Packer and Armitage (1994) using different chemoattractants. At this stage it is not possible to identify the effector molecule that links metabolism and the chemotactic response.

In a few other microorganisms, transport of a metabolite has been observed to be necessary for chemotaxis. This appears, for instance, to be the case for 4-hydroxybenzoate in *Pseudomonas putida* and L-proline in *E. coli* (Clancy *et al.*, 1981; Harwood *et al.*, 1994). The phosphoenolpyruvate transport system chemotactic cascade is also dependent on transport, but requires, in addition, the Che proteins (Lengeler and Vogler, 1989). It is possible that CheA and CheY homologues are also involved in chemotaxis in *R. sphaeroides,* since their presence has been demonstrated (Packer and Armitage, 1994). However, double mutants of *E. coli* in which the methylation system is deleted to remove the adaptation response still show a tactic response to pyruvate similar to the one observed in *R. sphaeroides* (Stock *et al.*, 1989). This may suggest that the MCP systems identified in most motile bacteria mask an underlying system similar to that which occurs in *R. sphaeroides.*

Experimental procedures

Bacterial strains and growth conditions

The bacterial strains used (Table 2) are derivatives of *R. sphaeroides* 2.4.1. (Nano, 1984). Strains were grown under aerobic conditions at 30°C on Sistrom medium with succinate as the carbon source and ammonium chloride as the nitrogen source. When required streptomycin (Sm; 50 $\mu g\,ml^{-1}$), kanamycin (Km; 25 $\mu g\,ml^{-1}$) or tetracycline (Tc; 1 $\mu g\,ml^{-1}$) was added to the medium. To suppress revertants of the glutamate transport-deficient mutant, the toxic glutamine analogue γ-glutamyl-hydrazide was used at a concentration of 1 mM. *E. coli* strains were grown aerobically at 37°C on Luria–Bertani medium supplemented with tetracycline (12.5 $\mu g\,ml^{-1}$), kanamycin (25 $\mu g\,ml^{-1}$) or ampicillin (50 $\mu g\,ml^{-1}$).

Isolation of glutamate transport-deficient mutants

E. coli S17–1 containing the plasmid pSUP2021 (Table 2) harbouring transposon Tn*5* (Simon *et al.*, 1983) was conjugated aerobically with *R. sphaeroides* 4P1 on membrane filters placed on the surface of LB plates. After 15 h at 30°C, serial dilutions were plated on Sistrom succinate–ammonium plates containing γ-glutamylhydrazide. About 200 colonies growing in the presence of γ-glutamylhydrazide were further tested for growth with either glutamate (25 mM) or succinate and ammonium chloride as nitrogen and carbon sources.

Table 2. Bacterial strains.

	Relevant characteristics	Reference
Strain		
R. sphaeroides 2.4.1.	Wild type	Nano (1984)
R. sphaeroides 4P1	*E. coli lac*Y under control of unknown *R. sphaeroides* promoter; Sm^R	Nano (1984)
R. sphaeroides MJ7	L-glutamate transport-defective derivative of strain 4P1; Sm^R; Km^R	This work
R. sphaeroides MJ7GltP	Strain MJ7 with plasmid pGMJ100; Sm^R; Km^R; Tc^R	This work
E. coli S-17	*thi pro hsdR hsdM*$^+$ *recA*, chromosomal insertion of (RP4-2(Tc::Mu)(*km*::Tn*7*))	Simon *et al.* (1983)
Plasmid		
pGBT521	Ap^R; carrying the *gltP* gene of *E. coli*	Tolner *et al.* (1995)
pCHB500	Tc^R; expression vector for *R. sphaeroides* derived from pRK415 and pSH3	Benning and Somerville (1992)
pGMJ100	Tc^R; *Eco*RI–*Pst*I fragment of pGBT521 containing *gltP* gene cloned into pCHB500	This work
pSUP2021	Ap^R; Km^R; Cm^R; pBR325::*mob*::Tn*5*	Simon *et al.* (1983)

The 15 colonies that appeared only on plates containing succinate/ammonium chloride were tested for glutamate and proline transport activity.

Transport assays

Transport studies in cells were performed as described by Abee *et al.* (1989a) at 30°C. Cells harvested during logarithmic growth were washed twice in 50 mM potassium phosphate of the desired pH containing 5 mM $MgSO_4$ and 50 $\mu g\,ml^{-1}$ chloramphenicol and resuspended to a protein concentration of 5–10 $mg\,ml^{-1}$. Cells were stored on ice until use. For uptake experiments, [^{14}C-]glutamate was used at final concentrations of 0.94 μM. For the analysis of the effect of ionophores on glutamate uptake, cells were treated with EDTA as described by Elferink *et al.* (1986). Ionophores were used at final concentration of 4 μM.

Chemotaxis assay

The chemotaxis assay was performed according to Tso and Adler (1974). Cells were washed twice and resuspended in 10 mM potassium phosphate pH 7.1 to a concentration of 10^9 cells ml^{-1}. After starving by incubation in the dark at 4°C for 4 h, the cells were mixed with an equal volume of 0.4% agar containing 100 $\mu g\,ml^{-1}$ chloramphenicol and poured around plugs of 2% agar containing the chemoattractant. After 15 h (or 2–3 h), chemotaxis was observed as rings covered by cells formed around the plug containing the chemoattractant. Competing chemoattractants were included in the 0.4% agar at the concentrations indicated.

DNA manipulations

Mini- and large-scale preparations of plasmid DNA were obtained by the alkaline lysis method (Birnboim and Doly, 1979; Ish-Horowicz and Burke, 1981). Chromosomal DNA was isolated essentially as described (Leenhouts *et al.* 1990), except that mutanolysine was omitted. Strains were transformed after rubidium chloride treatment of the cells (Sambrook *et al.*, 1989). Other DNA techniques were performed as described previously (Dower *et al.*, 1988).

Protein determination

The protein concentration of whole cells was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

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